Differences in Mating Behavior Among Three Populations of *Anthocoris antevolens* (Heteroptera: Anthocoridae): A Comparison of Intra- and Interpopulation Crosses

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ABSTRACT Mating behavior was compared among three populations of *Anthocoris antevolens* White (Heteroptera: Anthocoridae). Two of the three populations are sympatric in the Yakima Valley, Washington, and are known to differ in length of the setae on the hemelytra and in characteristics of the male's genitalia. The third population occurred 120 km west of the Yakima populations. All possible inter- and intrapopulation crosses were studied. Males attempted to mate females in all crosses and were as rapid in initiating mating attempts in interpopulation crosses as in intrapopulation crosses. Mating success, defined to be insemination of the female, varied between 64 and 92% in intrapopulation crosses, but only between 0 and 21% in interpopulation crosses. The crosses between the two sympatric populations never resulted in insemination. Females in all crosses resisted mating attempts by males. Resistance behavior included hunching of the abdomen to prevent insertion of the clasper by the male, use of a hind leg to block male attempts to insert the clasper, and attempts to dislodge the male. For two of the populations, female resistance increased when paired with a male from a different population than when paired with a male from her population; females from the third population showed similar levels of resistance in interpopulation and intrapopulation crosses. Our results support the idea that *A. antevolens* is actually composed of an unknown number of reproductively isolated cryptic species.

KEY WORDS Insecta, female resistance, insemination, cryptic species, geographic variation

THE PREDATORY BUG *Anthocoris antevolens* White (Heteroptera: Anthocoridae) is the most geographically widespread species of *Anthocoris* in North America and is found throughout Canada, south at least into the New England states in the eastern United States, north into Alaska, and south at least into Arizona and Baja California in the west (Anderson 1962, Kelton 1978, Henry and Froeschner 1988, Scudder 1997). The insect associates with a number of deciduous trees and shrubs, especially species in Salicaceae and Rosaceae (summary in Horton et al. 2004), and is a common insect in deciduous fruit orchards where it preys on soft-bodied arthropod pests (Westigard et al. 1968, Messing and AliNiazee 1985). *A. antevolens* exhibits substantial geographic variation among populations in size and shape of the male's genitalia, body size, and pubescence (Horton and Lewis 2005; unpublished data). Population divergence in these characteristics can occur even between sympatric populations (Horton and Lewis 2005). Moreover, the divergence has been shown also to be associated with reproductive

incompatibility, characterized by a lack of insemination despite attempts by males to mate females (Horton and Lewis 2005). These observations suggest that *A. antevolens* may be composed of a complex of cryptic species that are often fairly similar in outward appearance.

Horton and Lewis (2005) showed that a population of *A. antevolens* in the Yakima Valley, Washington, collected from oak, *Quercus garrayana* Douglas (Fagaceae), was reproductively isolated from a population collected from nearby stands of willow, *Salix* spp. (Salicaceae). Males from the two sources also differed in size and shape of the genitalia. Further examination of the *Salix* population showed that males from this source exhibited substantial variation in length of the phallus, leading Horton and Lewis (2005) to speculate that the population of *A. antevolens* inhabiting *Salix* in the Yakima Valley may itself actually comprise two distinct groups. Here, we describe aspects of mating behavior in these two putatively distinct populations of *A. antevolens* that associate with *Salix* spp. in the Yakima Valley and include for comparison a third population also collected from *Salix*, but inhabiting a site 120 km from the Yakima Valley location. We compared insects from the three populations for several characteristics, including copulation duration, time

required by the male to initiate mating attempts, insemination success, and female resistance to male mating attempts. Both intrapopulation and interpopulation pairings were made, allowing us to test whether insemination success was higher in intrapopulation crosses than in interpopulation crosses. We also quantified several types of female resistance behavior noted during the behavioral assays and compared populations for these traits. Last, we specifically tested the hypothesis that female resistance to mating attempts by the male would be more pronounced in interpopulation crosses than intrapopulation crosses. We have preliminary data showing that certain population crosses in *A. antevolens* that do lead to successful insemination may nonetheless result in production of infertile eggs (see *Discussion*). Thus, mating with certain males could potentially entail a fitness cost for females, and we anticipated that females would resist mating attempts by males from other populations.

Materials and Methods

Source of Insects and Rearing. Adults and nymphs of *A. antevolens* were collected from three sites. Twenty-five to 40 adults and nymphs were collected from each site, of which 10–15 mated females per site were used to begin laboratory cultures. The Alder Lake population (AL) was collected 21 June 2002 from several stands of *Salix* growing along a 20-km stretch of Highway 7 between Mineral Lake and Alder Lake, Washington (46° 71′ N, 122° 22′ W). A second population of bugs (GC) was obtained 14 June 2002 from ≈20 pear trees growing on the Apple Tree Golf Course in western Yakima, WA (46° 57′ N, 120° 61′ W), 120 km east of the AL site. The third population (UG) was collected 10 June 2002 from a 100-m stretch of *Salix* growing along the Yakima River just southeast of Union Gap, WA (46° 52′ N, 120° 47′ W). The UG and GC sites are separated by 12 km. However, we have recently found UG and GC insects co-occurring in late summer and fall on the same stands of *Salix* growing near Union Gap and, to a lesser extent, on poplars (*Populus;* Salicaceae) growing near the Golf Course site; thus, the two populations occur in definite sympatry. The UG and GC bugs differ in length of the setae on the hemelytra, and with use of a microscope can be differentiated on this basis (T.M.L., unpublished data).

Insects were reared in plastic Tupperware containers at 22–24°C and a photoperiod of 16:8 (L:D) h. The three populations were kept in separate environmental chambers. Pear seedlings that had been infested with eggs and nymphs of pear psylla, *Cacopsylla pyricola* (Förster), were provided to each culture. Pear psylla is a suitable food for *A. antevolens* from all three populations. The seedlings were used as oviposition substrates for the bugs. Nymphs were removed from each culture as late instars and moved individually to plastic petri dishes (9 cm in diameter) lined with filter paper. The insects were provided daily with freshly detached pear leaves that had been infested with pear psylla eggs and nymphs. Virgin adults, 2–5 d old that

had eclosed in the petri dishes, were used in all behavioral assays. The majority of the assays used offspring of the field-collected insects. A modest number of assays (<5% of total) late in the study necessarily used the F2 laboratory generation, to obtain the desired sample sizes.

Assay Methods. Mating assays were done using methods described previously (Horton et al. 2001, 2002). Assays were done in 6-cm-diameter plastic petri dishes at 22–24°C under fluorescent lighting. Females were placed individually in the petri dishes and allowed to settle for 15 min, after which a male was added to the dish. All possible combinations of inter- and intrapopulation crosses were done (nine possible crosses). Sample sizes were 25 pairs per cross. We monitored one to six pairs at a time, depending upon availability of bugs of the appropriate age.

Assay duration was 30 min per pair. For each pair, we recorded whether the male attempted to mate the female, the amount of time after the beginning of the assay at which the initial mating attempt was made, and duration of copulation. A male attempting to mate a female first mounted the female and then curled the end of his abdomen beneath the female's abdomen. He then attempted to insert his sclerotized clasper into the opening of the female's copulatory tube, located on her ventral surface near the opening of the oviduct. The clasper seems to be used by the male to pry open the entrance of the female's copulatory tube and to act as a channel into the copulatory tube for his membranous phallus. Intromission is defined here as insertion of the clasper. We used a hand-lens to confirm intromission. Copulation duration was defined as the interval between insertion of the clasper combined with settling of the male on the female's back, and withdrawal of the genitalia. We compared copulation duration among populations using only data for intrapopulation crosses that led to insemination (see below for methods used in determining whether the female had been inseminated), given the lack of insemination success in the interpopulation crosses (see below).

To provide more details on specific mating behaviors and to quantify the time course of these behaviors, we also visually scanned each pair at 2-min intervals ("instantaneous scan"), producing 15 scans per pair over the duration of the 30-min assay. At each scan, we classified male and female activities into one of five categories: 1) Not in physical contact. At the beginning of the assay (before initial contact of male and female) and after postcopulatory disengagement, the male and female often were not in physical contact. 2) Male mounted, attempting intromission. The mounted male had his abdomen extended beneath the female, but he had not successfully inserted his clasper. 3) Female resistance. This behavior included three distinct female activities that seemed to make it difficult for the male to achieve intromission: a) Hunching. The female's back was hunched, which had the effect of masking or covering the opening of her copulatory tube with an abdominal sclerite; b) Blocking. The female was using her right hind leg to block the male's

attempt to bring his clasper into contact with the opening of her copulatory tube; c) Attempting to dislodge male. The female was attempting to dislodge the male by rubbing or scraping the mounted male against the side of the petri dish as she rapidly moved around the dish. Note that categories 2, "male mounted, attempting intromission," and 3, "female resistance," are not mutually exclusive, because female resistance could occur only if the male was actually mounted. 4) Copulation. The male had inserted his genitalia and was quietly settled on the female's back. 5) Postcopulatory riding by male. After copulation or attempts at copulation, the male had removed his genitalia from the copulatory tube, but he had not dismounted.

At the end of each 30-min assay, the female was dissected and her sperm pouch was examined for the presence of sperm (Horton and Lewis 2005). Successful mating is defined here as the presence of sperm in the sperm pouch.

Voucher specimens for each population have been deposited in the M.T. James Museum, Department of Entomology, Washington State University, Pullman.

Statistical Analyses. To determine whether level of female resistance depended upon source of the male, we used a factorial (female population × male population) analysis of variance (ANOVA) on the cumulative number of scans in which female resistance was observed over the 15 instantaneous scans per pair. A square-root transformation was used on the data before conducting the ANOVA. To determine whether proportion of females exhibiting resistance behavior was affected by type of cross, we used multiway categorical analysis (Fienberg 1983). The analysis is analogous to the factorial ANOVA, except that the response is categorical (i.e., female did versus did not exhibit the behavior). Both tests were done to determine whether female resistance was more pronounced in interpopulation crosses than intrapopulation crosses. Effects of type of cross on copulation duration and time required for the male to initiate mating activities were analyzed with ANOVA.

Results

Male Mating Attempts, Insemination Success, and Copulation Duration in Intra- versus Interpopulation Crosses. Mating success (i.e., percentage of insemination) was higher in intrapopulation crosses (range 64–92% of males that attempted to mate the female) than interpopulation crosses (range 0–21%), despite efforts by males to mate with females in both types of crosses (Table 1). The 21% success occurred in the GC male × AL female cross, and it may be of interest that males in these two populations are the most similar in phallus length (unpublished data); however, the success was asymmetrical, in that the AL male × GC female cross was unsuccessful (Table 1). Pairings between the two sympatric populations (GC and UG) never led to insemination.

Initial attempts by males to mate the female occurred on average ≈8 min into the assay (range of the

Table 1. Results of mating assays showing frequency of insemination success, lack of success despite attempts, and lack of attempts for intra- and interpopulation crosses

	Insemination	Male attempts but no insemination	No attempt by male
GC male crossed with			
GC female	23 (92%)[a]	2	0
UG female	0 (0%)	18	7
AL female	5 (21%)	19	1
UG male crossed with			
GC female	0 (0%)	22	3
UG female	19 (86%)	3	3
AL female	0 (0%)	23	2
AL male crossed with			
GC female	0 (0%)	23	2
UG female	1 (5%)	20	4
AL female	14 (64%)	8	3

$n = 25$ pairs per assay.
[a] Percentage of attempted matings leading to insemination.

nine means 6.5–8.9 min [SEM = 0.9–1.8]). Means were similar among the nine crosses (by factorial ANOVA: female source, $F_{2, 180} = 0.04$, $P = 0.96$; male source, $F_{2, 180} = 0.18$; $P = 0.84$; interaction, $F < 0.1$). That is, males were as rapid in initiating mating attempts in interpopulation crosses as in intrapopulation crosses. Copulation duration in successful intrapopulation crosses was highly variable among pairs, irrespective of population source (range 3.3–16.2 min). Mean duration was similar among the three intrapopulation crosses (GC, mean = 8.8 [± 3.9 SEM] min; UG, 9.3 [3.5] min; AL, 9.7 [2.7] min; $F_{2, 52} = 0.3$; $P = 0.71$).

Time-Course Patterns of Behaviors in Intra- versus Interpopulation Crosses. For each of the 15 instantaneous scans per pair, we plotted the summed number of pairs (of the 25 pairs per cross) in which each activity was recorded at a given instantaneous scan; these summed frequencies are presented as area plots (Fig. 1). Total frequency of activities at a given instantaneous scan often summed to >25 pairs because "female resistance" and "male mounted, attempting intromission" are not mutually exclusive activities. Intrapopulation crosses are shown along the top row of Fig. 1.

For all crosses, the "not in physical contact" category occurred at highest frequency (gray fill in Fig. 1). The behavior "males mounted, attempting intromission" (diagonal slashed area just above gray fill) was noted in at least some pairings throughout the 30-in assay except late in the assay in the UG female × male cross (Fig. 1H). "Female resistance" (white fill just below black fill in each panel) tended to be less pronounced in the intrapopulation crosses than the interpopulation crosses, particularly for females from the AL and GC populations. Formal statistical tests of this observation are provided below.

"Copulation" (black fill in Fig. 1) was observed in virtually all scans in all crosses, except for late in the assay in the UG female × AL male cross (Fig. 1H); as noted above, there also were no mating attempts by males (diagonal slash) late in the assay for this par-
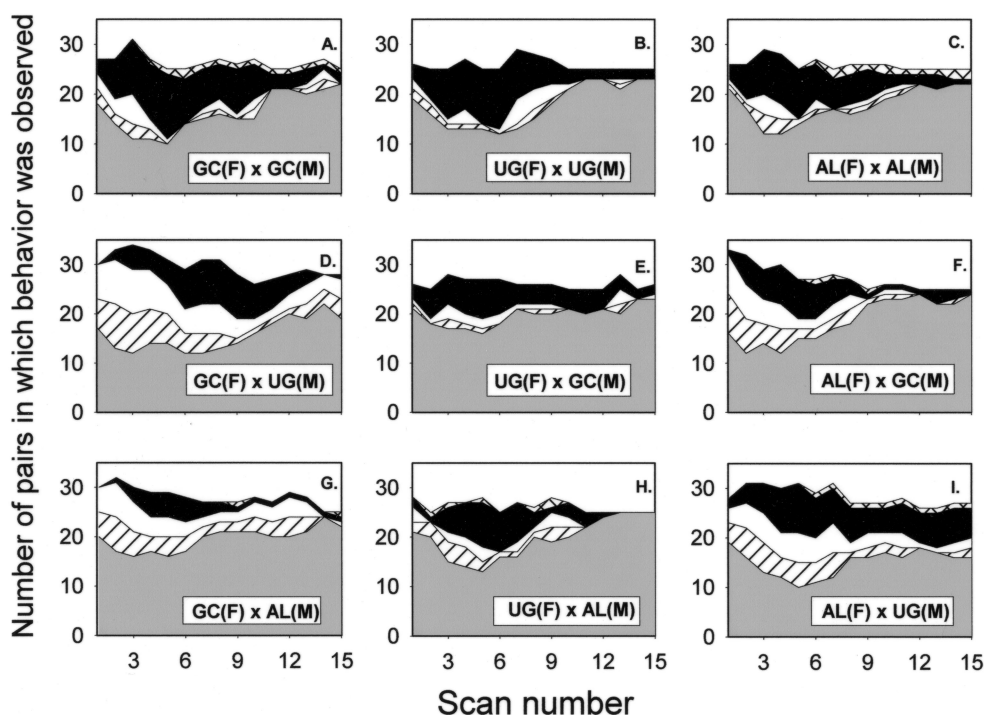
Fig. 1. Area plots showing number of pairs exhibiting a given behavior plotted as a function of scan number (15 instantaneous scans per pair); $n = 25$ pairs per cross. Intrapopulation crosses shown in panels on top row; interpopulation crosses shown in panels in bottom two rows. Summed frequency may exceed 25 at a given scan because two categories ("male mounted, attempting intromission" and "female resistance") are not mutually exclusive and could both be observed for a given male/female pair during a given instantaneous scan. Gray fill, "not in physical contact"; diagonal slash above gray fill, "male mounted, attempting intromission"; white fill above diagonal slash, "female resistance"; black fill above white fill, "copulation"; and cross-hatch fill above black fill, "postcopulatory riding by male."

ticular cross. "Postcopulatory riding by male" (cross-hatched fill above black fill) occurred in a small percentage (<8%) of pairings. The behavior was seen even in a few interpopulation crosses (Fig. 1G and I) for which insemination was never observed (Table 1).

Types and Levels of Female Resistance in Intra- versus Interpopulation Crosses. Female resistance activity in Fig. 1 consisted of three common behaviors, which we have shown for each of the nine crosses (Figs. 2 and 3). Figure 2 indicates percentage of females showing the behavior in at least one of the 15 instantaneous scans made during the 30 min assay. Figure 3 expresses the total number of scans per female in which a given behavior was noted. Means were calculated by first summing (for a given female) the number of scans in which the behavior was seen during the 15 instantaneous scans and then averaging the summed frequencies for that particular cross. Results in Figs. 2 and 3 exclude females for which the male did not attempt to mate the female, because without a male attempt there could be no female resistance (thus, sample sizes for each cross can be found in Table 1 by subtracting the number of nonattempts from 25). The arrows in Figs. 2 and 3 are provided to indicate the intrapopulation crosses.

Of the three types of resistance behavior, hunching and attempts to dislodge tended to occur with higher frequency than blocking (Figs. 2 and 3), although not in all crosses (e.g., blocking was relatively common in the AL female × GC male cross). If resistance behavior is pooled into a single category ("all resistance behavior"), results suggest that source of males had much lesser effect on female resistance in pairings involving UG females than pairings involving GC or AL females (Figs. 2 and 3). Formal tests of this observation are provided below.

We hypothesized at the beginning of this study that resistance behavior in females would be more pronounced in interpopulation crosses than intrapopulation crosses. Support for this hypothesis would require that the bars for intrapopulation crosses in Figs. 2 and 3 (those bars indicated by arrows) would be lower than the other two bars in any particular trio of bars. Statistical support would include a significant female population × male population interaction in the statistical test, followed by a significant male population effect within each female population. We used this approach to analyze the data on "all resistance behavior" (Figs. 2 and 3). In both analyses, the female population × male population interaction was significant
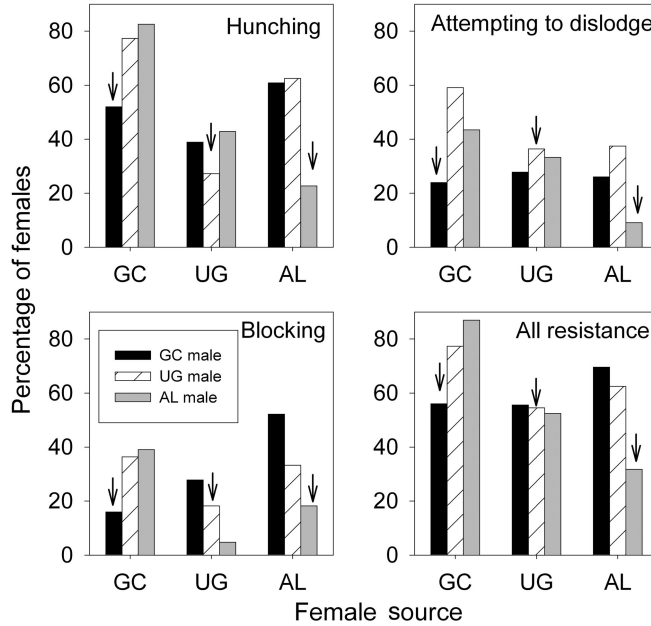
Fig. 2. Percentage of females in which resistance behavior was observed in at least one of the 15 scans as a function of type of cross. Arrows indicate the intrapopulation crosses. Excludes pairs in which the male did not attempt to mate the female.

(Table 2, test 1), indicating that the effects of male population on female resistance were not equivalent across all female populations. Because of the significant interaction, we conducted statistical tests for each female population separately (Table 2, test 2). These analyses showed that male population affected

level of female resistance for the GC and AL females, whereas resistance in UG females was not affected by male source (see also Figs. 2 and 3). A final analysis (Table 2, test 3) showed that resistance by GC and AL females was significantly more pronounced in inter-population crosses than in intrapopulation crosses
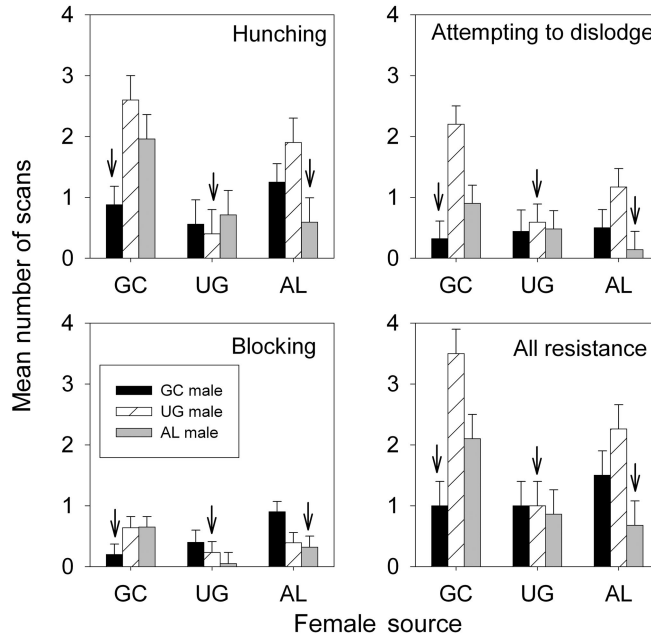

Fig. 3. Mean (SEM) number of scans in which the female was observed resisting the male. Means were calculated by first summing the number of scans (over the 15 scans for each female) that a behavior occurred for a given female and then averaging (over females) the sums. Excludes pairs in which the male did not attempt to mate the female.

Table 2. Summary of statistical tests on the role of male and female source affecting female resistance behavior

Test		% females (Fig. 2): categorical analysis			Mean no. of scans (Fig. 3): ANOVA[a]		
		χ^2	df	P	F	df	P
1, Is female pop × male pop interaction significant?		11.5	4	0.022	3.3	4,191	0.013
2, For a given female pop, does resistance depend upon male pop?	GC female	6.1	2	<0.05	5.9	2, 67	0.004
	UG female	0.1	2	<0.95	0.2	2, 58	0.86
	AL female	7.2	2	<0.03	3.4	2, 66	0.039
3, For a given female pop, is resistance lower in the intrapopulation cross than in the two interpopulation crosses?	GC female	5.5	1	<0.025	9.5	1, 68	0.003
	UG female	0.0	1	<0.99	0.1	1, 59	0.76
	AL female	7.0	1	<0.01	6.5	1, 67	0.013

Excludes pairs in which male did not attempt to mate the female. Statistical tests done for "all resistance behaviors" in Figs. 2–3.

[a] Data square-root transformed before analysis.

(Figs. 2 and 3), which is consistent with the hypothesis advanced at the beginning of the study.

Discussion

Intraspecific geographic variation in behavior of insects, including sexual behavior, has been less thoroughly studied than geographic variation in traits such as morphology, physiology, and life history characteristics (Foster and Endler 1999). Yet, studies of geographic variation in behavior, particularly when coupled with studies of other biological processes, or with genetic or morphological studies (Porter and Shapiro 1990, Arnqvist and Thornhill 1998), are useful in understanding aspects of speciation and species diversity (Foster and Endler 1999, Verrell 1999). These sorts of complementary studies have been particularly useful in understanding speciation and diversity in taxonomically difficult groups such as *Drosophila* (Diptera), and the studies have been used to help differentiate among closely related and morphologically similar sibling species (Coyne 1983, Coyne and Orr 1997, Civetta and Singh 1998).

Our results indicated that male *A. antevolens* vigorously attempted to mate with females from different populations (Table 1; Fig. 1), irrespective of male source. A cursory look at mating activities or external morphology in these bugs in the absence of previous knowledge that the populations differed significantly in genital morphology (Horton and Lewis 2005) would likely not have led us to conclude that there were uncertainties about reproductive isolation in these bugs. Dissection of the female's sperm pouch was necessary to demonstrate that bugs from the different populations were often reproductively isolated from bugs in other populations. Price et al. (2001) studied sperm transfer between closely related and cryptic species of the *Drosophila simulans* Sturtevant complex and concluded that reproductive isolation could not be inferred merely by watching copulation between closely related groups of flies, because copulation often failed to result in sperm transfer. Dissection of the female was necessary to demonstrate isolation. The authors used the term "cryptic reproductive isolation" to describe this phenomenon, and it

seems that this is appropriate terminology to use in the current study as well.

Females from all three populations resisted male attempts at mating by hunching the abdomen, blocking with the hind leg, and attempting to dislodge the male. These activities occurred in both intrapopulation and interpopulation crosses, although for the AL and GC females resistance levels were highest in the interpopulation crosses (Figs. 2 and 3; Table 2). That most females resisted male attempts to mate is not unexpected, because female resistance behavior is common throughout the Insecta (Richards 1927, Thornhill and Alcock 1983). Twisting of the abdomen away from the male's genitalia, as seen here in *A. antevolens*, seems to be a fairly common strategy to prevent intromission among a number of insect taxa (Thornhill and Alcock 1983). The increased resistance noted in AL and GC females paired with a male from a different population is of interest and may reflect attempts by the female to avoid insemination by a completely inappropriate male. We have obtained eggs from AL females that had been inseminated by GC males (i.e., the same interpopulation cross for which there was modest success by males; Table 1), but the eggs invariably failed to hatch, suggesting that there is also postinsemination isolation between at least some populations of these bugs (unpublished data).

Despite female resistance, males in interpopulation crosses did often manage to insert their claspers, thus we recorded some fraction of pairs for interpopulation crosses as being in copulation in virtually each of the 15 instantaneous scans (black fills in Fig. 1). This raises the question of why almost no males were able to inseminate the female in the interpopulation crosses. We have speculated elsewhere (Horton and Lewis 2005) that population differences in genitalia of male *A. antevolens* might lead to reductions in insemination success. By flash-freezing pairs in the act of copulation, we showed that males in certain interpopulation crosses often had failed to fully inflate the phallus, unlike the condition that was seen in males from intrapopulation crosses (Horton and Lewis 2005). We assume, but have not demonstrated, that the phallus must be fully inflated for the male to inseminate the

female. The only success in insemination seen in the interpopulation crosses involved the two populations (AL and GC) in which males from the two populations are the most similar in phallus length (unpublished data). Whether this morphological similarity was responsible for the insemination success in this cross remains to be addressed more directly.

A. antevolens is a geographically widespread species in North America occurring on a variety of deciduous trees and shrubs. Questions were raised years ago about the taxonomic status of *A. antevolens*, because of similarities in biology, appearance, and shape of the male's clasper between it and a mostly eastern species, *Anthocoris musculus* (Say) (Hill 1957, Anderson 1962). That is, questions were raised as to whether *A. antevolens* and *A. musculus* were distinct species. Results reported here and previously (Horton and Lewis 2005) suggest that the situation is even more complex than thought by Hill (1957) and Anderson (1962), in that *A. antevolens* may actually be composed of a complex of morphologically similar populations that differ in certain characteristics, including male genitalia (Horton and Lewis 2005) and sexual behavior (e.g., compare females from the sympatric UG and GC populations in resistance behavior, Figs. 2 and 3). The variation in genitalia and behavior extends to include sympatric populations now known to be reproductively isolated (Horton and Lewis 2005; this study, Table 1), suggesting that *A. antevolens* is a complex of an unknown number of cryptic species.

Acknowledgments

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